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The distribution of beneficial mutations and their pleiotropic effects

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THE DISTRIBUTION OF BENEFICIAL MUTATIONS AND THEIR PLEIOTROPIC EFFECTS.

BY

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The Distribution of Beneficial Mutations and Their Pleiotropic Effects

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Understanding the distribution of beneficial mutations is relevant to the understanding of virtually all aspects of the adaptive process. Data describing this distribution are limited, but even less well understood are the pleiotropic effects of adaptive mutations. Yet, the pleiotropy of beneficial mutations may play a role in numerous biological processes. In this thesis, we address this problem by measuring the distribution of beneficial mutations and their pleiotropic effects using mutants of *Burkholderia cenocepacia* and *Escherichia coli.* The fitness of each mutant was measured relative to its progenitor in several novel environments. We found that the effects of beneficial mutations are best explained by an exponential distribution. We also found that most beneficial mutations increased fitness in alternative environments, indicating positive pleiotropy. Thus, the early steps of adaptation appear to generally expand niche breadth, with mutations of larger benefit producing greater improvements in foreign environments.

CHAPTER I

INTRODUCTION

The distribution of adaptive mutations

Amongst all types of mutations, the small fraction of mutations that are beneficial generates adaptation, and the precise distribution of effects among these beneficial mutations is of broad biological importance of its relevance to the rates of adaptation (Sanjuan et al., 2004a) population bottlenecks (Wahl et al., 2002) and convergent evolution (Colosimo et al., 2005, Peichel, 2005). effects of population size, the dynamics of adaptation, and the likelihood of convergent evolution. If mutations of small benefit are much more common than those of greater benefit, then adaptation occur gradually, as these small benefit mutations incrementally assemble. However, as mutations of larger benefit become more abundant, adaptation may occur more rapidly by greater steps (Wilke, 2004, Fisher, 1930). The distribution of mutational effects also influences whether the path of adaptation is predictable. For example, beneficial mutations with only a slight fitness advantage are often lost due to genetic drift or by competition between clones, also known as clonal interference (Gerrish 1998). Both processes are influenced by stochastic events and thus adaptation by small steps is more likely to occur differently in replicate populations, though less so in

large populations whose size produces more deterministic outcomes (Rozen et al., 2002). In general, beneficial mutations with large effects are assumed to be rare relative to beneficial mutations with small effect (Fisher, 1930). The distribution of beneficial mutations was first suggested to be exponential by Gillespie (Gillespie, 1983, Gillespie, 1984). Orr (Orr, 2003) showed theoretically that the distribution of beneficial mutations is exponential using the extreme value theory (EVT), which is a body of probability theory that is concerned with the properties of draws from the tails of distributions (Gumbel, 1958, Leadbetter et al., 1983). If beneficial mutations are rare, then the relevant portion of the full fitness distribution is the extreme right tail, especially in larger populations (Rokyta et al., 2008). As population size decreases, however, the shape of this rare tail becomes increasingly important to explain stochastic differences in adaptive paths among different populations. Further, the distribution also predicts the spacing of effects between beneficial mutations that are likely to be fixed (Orr, 2003), and hence the rate and pattern of adaptation.

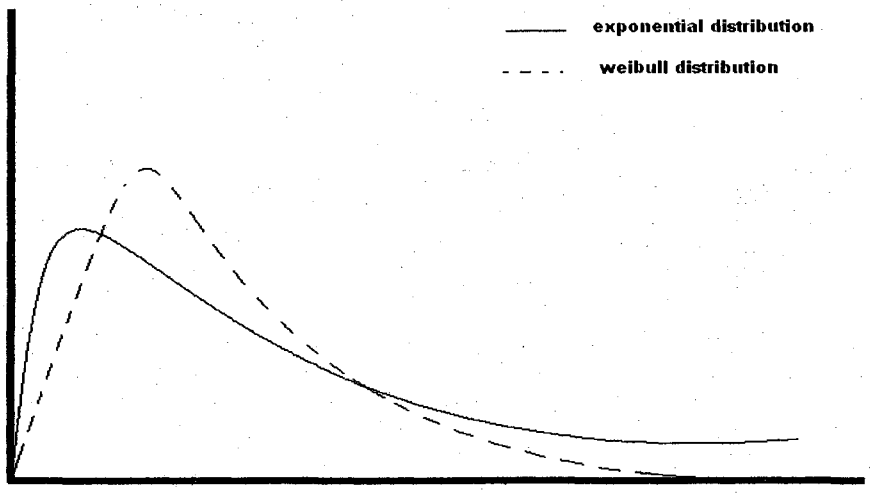


Figure 1. Graph of two distributions, exponential and Weibull. Experimental evidence supports that the distribution of beneficial mutations may fit one or the other

Empirical measures of beneficial mutations sorted by their rank order of effects appear to follow an exponential distribution (Sanjuan et al., 2004b, Kassen & Bataillon, 2006, Imhof & Schlotterer, 2001). However, recent theoretical and empirical work demonstrating that the distribution of beneficial mutations in two collections of viruses is truncated at its rare, most fit limit (known as a Weibull domain of attraction) (Rokyta et al., 2008). Rokyta et al. used two sets of bacteriophage mutants derived from ID11 and Φ6 that were selected for increased growth rate and novel host range respectively. Rokyta et al. argue that more collections of beneficial mutations are needed to define the shape of the rare tail of the distribution of mutational effects.

The distribution of pleiotropic effects

Pleiotropy, defined as a single mutation that affects multiple phenotypes (Ostrowski et al., 2005), has been implicated in countless biological processes such as antibiotic resistance (Lenski, 1998), aging (Williams, 1957), and specialization (Cooper & Lenski, 2000). For a process with so many biological implications, very little is known about the distribution and abundance of the pleiotropic effects of mutations, especially for beneficial mutations. The relationship between genotype, phenotype, and adaptation has been demonstrated several times theoretically, but only a handful of times experimentally (Bulmer, 1973, Barton, 1990, Cooper & Lenski, 2000, Cunningham et al., 1997, Dudley et al., 2005, Fisher, 1930, Gerrish & Lenski, 1998, Gillespie, 1984, Griswold & Whitlock, 2003, Lande, 1980, Lenski, 2004, Orr, 2003, Ostrowski et al., 2007, Otto, 2004, Turner & Chao, 2003, Velicer et al., 2006, Wichman et al., 1999). The theoretical studies that have tried to examine the effects of pleiotropy on mutations rely on a common set of assumptions, mainly being that the distribution of pleiotropic effects is unknown (Otto, 2004). For example, it is unclear if the first steps of adaptation to one environment affect fitness only in that environment, or alternatively, if these first steps affect fitness in a range of environments, potentially producing trade-offs and a narrower niche. This specialization to a single environment can also reduce the fraction of mutations that can produce beneficial alleles (Otto, 2004), meaning that well-adapted organisms can acquire fewer beneficial mutations than less well-adapted organisms.

The distribution of pleiotropic effects among mutations has been studied experimentally by two different approaches. In one, a genome is saturated with mutations and their effects are measured in several environments. An example of this type of approach, growth phenotypes of a collection of mutants with deletions in the non-essential open reading frames in *Saccharomyces cerevisiae* were measured in 21 different experimental conditions (Dudley et al., 2005). Of the 4710 mutants collected, 767 of these mutants displayed significant growth defects, indicating negative pleiotropy when compared to the control under at least one of the 21 conditions. The authors concluded that a greater degree of pleiotropy was observed than would have been seen by chance, and concluded that pleiotropy was widespread (Dudley et al., 2005). An advantage of this type of study is that the pleiotropic effects of most of the genes in the genome can be sampled, but a disadvantage is that these mutations were neither naturally arising nor favored by selection.

In second type of approach, a population is exposed to a specific environment that selects for advantageous mutants that are captured and assayed in both selective and alternative environments. In an example of this latter type of approach, 665 random mutants of *Pseudomonas fluorescens* that were resistant to the antibiotic nalidixic acid were collected using "hard selection". Once collected, their effects were evaluated in the selective environment and three alternative environments (Kassen & Bataillon, 2006). Most of the mutants collected were deleterious for fitness (how well a mutant reproduces over a set period of time against its ancestor), but 28 of the mutants had increased growth

rate when compared to the wild-type in permissive conditions (wild-type would not grow in the selective condition). When the 95 highest ranked mutants are plotted, the distribution of fitness effects are exponential for all environments; furthermore, the mean fitness effect of these mutants is statistically invariant across all environments, which suggests positive pleiotropy, or that these single adaptive mutations lead to higher fitness in the all tested environments (Kassen & Bataillon, 2006). This is notable because theory suggests that the mean fitness effects of a population of mutations depends on the size of the fitness gap between the first and second most fit mutant (Orr, 2003). The size of this gap seems likely to change between environments, which means that the mean fitness should vary (Kassen & Bataillon, 2006).

One advantage of this latter study is that the environment naturally selects for the mutations that increase fitness. The major disadvantage is there may be limited targets to confer naladixic acid resistance and the wild-type does not grow in the selective environment, making it impossible to directly compare a mutant with the wild-type to measure fitness. The authors concluded that the distribution of beneficial mutations adheres to the popular model that big benefit mutations are rare, while smaller benefit mutations are common, and that the distribution of fitness effects does not vary with environment (Kassen & Bataillon, 2006).

In contrast to the hard selection of antibiotics, another set of studies used "soft selection" to favor mutations that improve the fitness of populations of *Escherichia coli* growing in simple laboratory conditions. Thirty populations of *E. coli* were grown for up to 400 generations until a beneficial mutant rose to a

detectable frequency in the population. The direct benefits of these mutations were first measured in the selective environment (Rozen et al., 2002) and then their pleiotropic effects were assayed in five different carbon sources (galactose, meliobiose, N-acetylglucosamine, maltose, and mannitol (Ostrowski et al., 2005). Because carbon sources differed in their mechanisms of uptake, they were expected to illustrate trade-offs associated with resource-specific adaptation. However, pleiotropic effects of these mutants were generally positive, save for one resource, which implies that the early steps of adaptation improve fitness in a range of environments and hence broadened the potential niche.

One possible caveat of this finding of abundant positive pleiotropy, as the authors themselves noted, is that selection may have acted to improve resource uptake in general, which would enhance fitness mostly independently of the specific carbon source but that may harm fitness in environments differing by another factor (e.g. temperature, oxygen availability, etc.). Therefore, measuring fitness effects of these same mutants in more foreign environments is warranted. In addition, because the molecular targets of adaptation in these *E. coli* populations (Cooper et al., 2002) have been found to occur repeatedly in replicate populations, it is possible that the uniformity of pleiotropic effects may have been exaggerated by repeated sampling of the same mutation. A later study of these same mutants (Ostrowski et al., 2007) found that mutations at the same locus produced similar pleiotropic effects, while other mutants harboring different mutations at the same locus produced significantly different pleiotropic effects. This implies that the form and magnitude of pleiotropy need not always

correlate with the mutated locus. While these studies have begun to examine the biological relationship between mutation and pleiotropy, more studies are needed, especially since the results of these experiments have complicated rather than clarified matters.

Advantages of experimental evolution

Numerous organisms have been subjected to experimental evolution using a variety of designs, notably: viruses (Heineman & Bull, 2007, Miralles et al., 1999, Ferris et al., 2007), yeast (Dudley et al., 2005, Segre et al., 2006), and several types of bacteria (Riley et al., 2001, Lenski et al., 1991, Hegreness et al., 2006). Using small organisms with fast reproductive rates has not only allowed for rapid evolution in relatively short time increments, but it also has allowed for reproducibility. By having several replicate populations evolving at the same time, we are able to observe if adaptive evolution is predictable or influenced substantially by random processes. Experimental evolution has also provided insight into the costs of adaptation (Cooper & Lenski, 2000, Cooper et al., 2002) and its underlying mechanisms (Cooper et al., 2001).

Most of these experiments involve long-term evolution in which multiple adaptive mutations have fixed in sampled populations. While this approach has shed a great deal of light on how adaptation to a controlled environment occurs, the epistatic interactions between mutations makes it almost impossible to tease apart relationships between individual mutations and their phenotypes. Instead, a short-term evolution project in which an population only has one mutation rise

to a high frequency can shed light not only on the magnitude of the first steps of adaptation, but also the pleiotropic effects of single mutations: in particular, the effects of early adaptation on fitness in other environments.

In this thesis, four collections of mutants were used. Two collections were isolated from *Burkholderia cenocepacia* HI2424 as follows; In one collection, 16 mutants were isolated from populations founded by a single clone that evolved in an environment where galactose was the limiting carbon source. The second *B. cenocepacia* collection was collected by random transposon mutagenesis (Benton and Cooper, unpublished). The other two mutant collections were isolated from *Escherichia coli* in different ways. In one collection, 14 mutants were isolated from populations founded by a single clone that evolved in an environment where galactose was the limiting carbon source. In the second *E. coli* collection, 14 mutants were isolated from populations founded by a single clone that evolved in an environment where glucose was the limiting carbon source (Rozen et al., 2002).

Using these four mutant collections allows us to address two questions: 1) How are the effects of beneficial mutations distributed? and 2) How are pleiotropic effects related to beneficial effects? We also address other questions enabled by our design: i) How does the environment affect the distribution of beneficial and pleiotropic effects? By finding the distribution of beneficial mutations among mutant collections of the same bacteria that were evolved in different selective environments, we can begin to tease apart how the environment influences this distribution. ii) How common are genotype-by-environment interactions, and do

different organisms evolved in the same environment display different patterns? By utilizing the *B. cenocepacia* and *E. coli* mutants that were evolved in the same environment, we can determine if genotype plays a role in the distribution of pleiotropic effects and iii) How does the mode of mutant selection influence our interpretations of these questions? In the past, there have been two distinct types of mutant collections; those that let the environment select for beneficial mutations or those that artificially introduced mutations. By using a combination of these techniques, we have to ability to find if the results are the same, or if the mode of mutation influences the distribution of pleiotropic effects.

CHAPTER II

MATERIALS AND METHODS

Strains and culture conditions

The *E. coli* B used in this study was the founding strain of the Lenski long-term lines, REL 606. REL 606 ($T6^r$, Str^r, Ara^-) was passaged from many years in the laboratory before being frozen, so some adaptation to different laboratory environments occurred. This strain of *E. coli* has been cured of all plasmids and bacteriophages so it cannot undergo genetic exchange and contains a single chromosome. REL 607 is a spontaneous mutant of REL 606, and is exactly the same except for its ability to utilize arabinose (Ara^+) as a carbon source (Lenski, 2009).

The *Burkholderia cenocepacia* HI2424 isolate used in this study is a member of the *Burkholderia cepacia* complex (BCC). This bacteria was first described by William Burkholder in 1950 as the causative agent of sour skin in onions (Burkholder, 1950), and now the Bcc is comprised of 17 formally named species (Vanlaere et al., 2009). HI2424 was isolated in an onion field in upstate New York and has undergone little, if any laboratory adaptation. It also contains three chromosomes and a mega-plasmid that give *B. cenocepacia* considerable genetic and metabolic diversity (O'Sullivan & Mahenthiralingam, 2005, LiPuma et al., 2002). HI2424 is a clinically relevant isolate because of its potential to infect

immunocompromised individuals, specifically those infected with cystic fibrosis (Isles et al., 1984). The isolates of HI2424 that were used in this study carried two marker types. One isolate of HI2424 was marked with the plasmid pCELacZ (Ellis et al. 2009), which is based on a Tn7 delivery system conferring the phenotypes Tp^r and Lac^+. Another isolate of HI2424 was marked with the plasmid pTn7-FTP, which conferred only the phenotype of Tp^r.

Novel environments

Each novel environment was chosen so that it either 1) provided a carbon source that used a different transport system or 2) presented a stressor to each mutant not seen during the initial evolution. These novel environments are: trehalose, glucose, galactose, paraquat (oxidative stress), bathophenanthroline (iron limitation), novobiocin, and static culture.

Trehalose is a phosphotransferase system (PTS) sugar that uses the porin LamB to cross the inner membrane (Klein & Boos, 1993). While glucose is also a PTS sugar, glucose uses the OmpF porin to cross the inner membrane. Galactose is a non-PTS sugar, which like glucose, uses the OmpF porin to cross the inner membrane (Travisano & Lenski, 1996). Several studies have been performed comparing carbon source utilization amongst mutants (Travisano & Lenski, 1996, Ostrowski et al., 2005). These studies found that mutations that improve fitness in one carbon source, often improve fitness in alternative carbon sources leading to positive pleiotropy.

To complement environments differing in carbon source, other stressors were added to the selective environment. Paraquat is a viologen, which reacts with free electrons to from radical ions. Oxygen reconverts the free radicals, and in the process, gives rise to super oxides. These super oxides react with unsaturated lipid membranes, rapidly disintegrating cell membranes and tissues (Gil et al., 2007). Outer membrane porin OmpW has been implicated in resistance to paraquat (Gil et al., 2007). Novobiocin is an aminocoumarin from *Streptomyces niveus* (Donnelly & Blagg, 2008). Novobiocin targets the *gyrB* subunit by competitively inhibiting the ATPase activity catalyzed by GyrB, while resistance to novobiocin is mediated by the amount of phospholipids in the outer (Mdluli & Zhenkun, 2007, Cooper, 2002). Bathophenanthroline chelates with free Fe (II) and makes iron unavailable for uptake. Bathophenathroline can also form complexes with Ru (II) and Cu (I) (Cowart et al., 1993). Iron is essential for the growth in most microorganisms, acting as a cofactor for processes such as electron transfer and RNA synthesis (Braun, 1997) as well as a virulence factor for bacterial pathogens (Lamont et al., 2002).

Collection of mutants

Ten replicate cultures of both *B. cenocepacia* and *E. coli* were founded using equal numbers of oppositely marked ancestors. The culture volume of 150 µl was maintained in 96-well plates at 37°C, shaking at 150 rpm. These cultures were propagated daily by diluting 0.2 µl of culture into 150 µl of M9 minimal media supplemented with 1% galactose. This 750-fold dilution yielded

approximately 9.5 ($\log_2 750$) generations per day. The effective population size for *E. coli* is 5.8×10^4 while the effective population size for *B. cenocepacia* is 1.7×10^5. While being propagated, each replicate culture was plated every three days on their respective indicator agar to assess if a deviation in marker ratio occurred (tryptic soy broth containing X-gal for *B. cenocepacia* and tetrazolium and arabinose agar for *E. coli*). If a deviation in marker ratio was sustained for more than three consecutive passages, the evolution of that population was stopped and archived at -80°C. This deviation indicates a beneficial mutation arising in a subpopulation of the evolving cultures. If no deviation in marker types occurred by 500 generations, samples of both marker types of each population were selected and archived for further study. Evolution was not allowed past 500 generations to minimize the likelihood of sequential fixation of more than one adaptive mutation. This time scale was based on upon earlier theoretical work that provided estimates for beneficial mutation rates (Gerrish & Lenski, 1998) as well as experimental work (Rozen et al., 2002). Ostrowski et al. found that in a previously collected set of mutants (which were collected so that they should only contain a single beneficial mutation), one out of thirty mutants contained a double mutation (Ostrowski et al., 2007). It is important to note that in our experimental evolution, while we observed fixed beneficial mutations, we never saw a deviation in marker ratio. Instead, all populations were stopped from evolving at 500 generations. Since a deviation was not seen, it may be there was clonal interference not only between the two marked populations, but there

was also clonal interference between subpopulations in the same marker type, indicating that beneficial mutations were abundant in our selective environment.

To complement the mutants we collected by experimental evolution, two other mutant collections were used. The first is a collection of *B. cenocepacia* mutants isolated by transposon mutagenesis using an EZ-Tn5 Transposon (Epicentre) modified to contain a trimethoprim resistance cassette (Benton and Cooper, unpublished). Trimethoprim-resistant mutants were then screened for increased fitness in M9+1% galactose and 14 were selected for further analysis. The second collection of *E. coli* mutants has been previously described (Rozen et al., 2002), but is described briefly here. A single clone of *E. coli* B was evolved in Davis Minimal + 25ug/ml glucose for up to 400 generations in the selective environment, or until a beneficial mutation was detected. Once detected, the both the winning and losing populations were archived at -80°C for further analysis.

Statistical analyses

Distribution of direct effects

To determine the distribution of the direct effects, a least sum of squares regression was performed to fit a linear and an exponential distribution to the data. First, a histogram of each mutant collection was prepared, rounding the relative fitness of each mutant to the neatest tenth. Any mutant with a fitness value of less than one was excluded from the analysis. The least sum of squares

was performed in Microsoft Excel using the LOGEST and LINEST function for the exponential and linear fit, respectively.

Effects of genotype and environment

To determine the effects of environment and genotype, two-way analyses of variance (ANOVA) were performed using a multivariate generalized linear model (SPSS v.15.0). Fitness was treated as a fixed effect and environment and mutant were random effects. An ANOVA was performed on each mutant collection separately to determine if the fitness of mutants varied significantly and if mutant fitness varied among environments.

Similarity of adaptation to the selective environment

To determine if each mutant collection responded similarly in different environments, and thus may have shared similar mutations, a hierarchical cluster analysis was performed on mutant fitnesses in all environments (SPSS v. 15.0) using a normalized Euclidean distance with the cluster analysis being between-group linkage. This analysis was performed on each of the four mutant collections.

Fitness assays

The fitness of each mutant was assessed by head-to-head competition with the evolved mutant against its ancestor in each environment: galactose, glucose, trehalose, novobiocin, paraquat, and bathophenanthroline. Competition assays

were performed as previously described (Lenski et al., 1991), with three-fold replication, and summarized here. Each competitor was inoculated from freezer into 5 ml of LB (Bertani, 1951) and allowed to grow for 24 hours. Next, the overnight cultures were diluted 1:100 into the novel environments to be tested and allowed to acclimate to this environment for an additional 24 hours. The competition began when equal amounts of ancestor and mutant were mixed and diluted into fresh media containing the novel environment, and allowed to compete for 24 hours before being sampled again. Initial and final day cultures were plated on indicator agar to enumerate the colony forming units of each competitor. Fitness (W) was calculated using the following equation with m_i denoting the number of mutants at a given time and a_i denoting the number of ancestors at that time.

$$W = \frac{\mathrm{Ln}(m_{24}/m_0)}{\mathrm{Ln}(a_{24}/a_0)}$$

Pleiotropic index

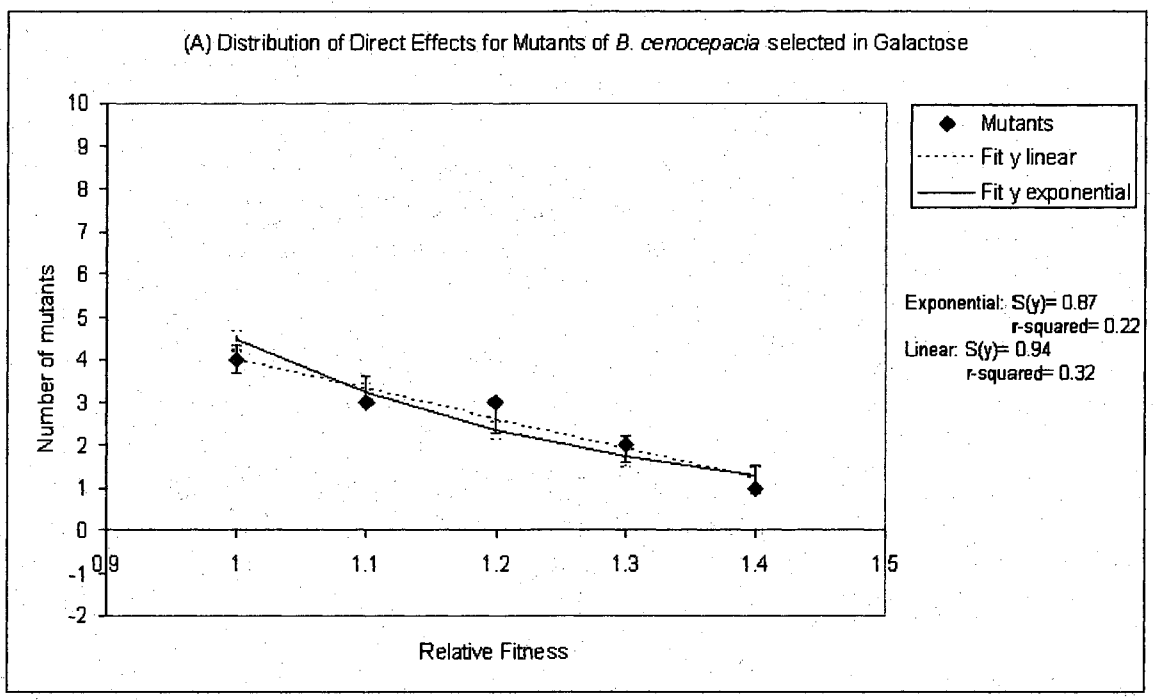
To summarize relative fitnesses in each novel environment for an individual mutant, a measurement called the pleiotropic index (PI) was used. The pleiotropic index is defined as the average of the deviation from ancestral fitness, which is by definition 1.0, in each novel environment, excluding the fitness value of the selective environment.

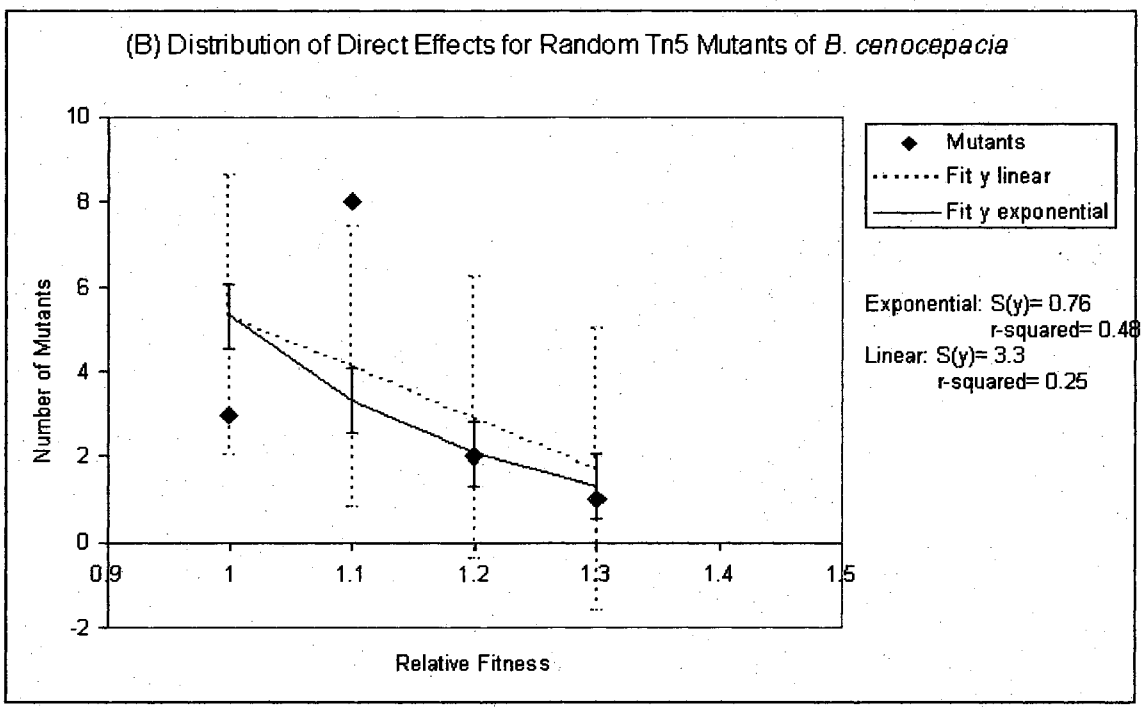
CHAPTER III

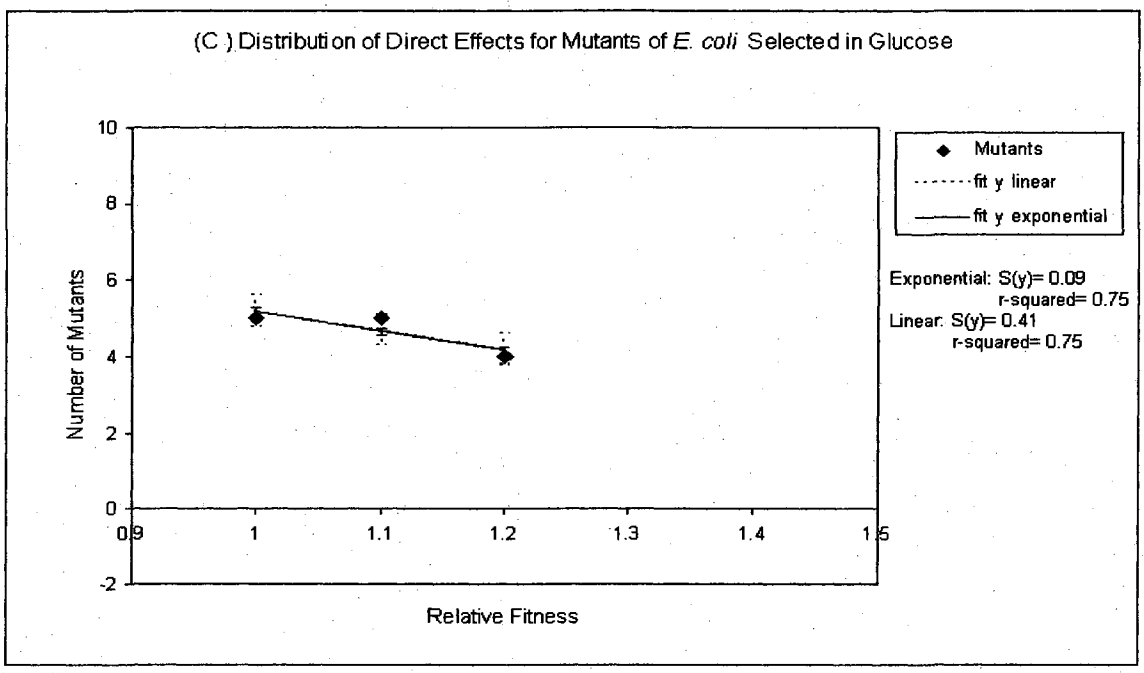
RESULTS

The distribution of beneficial mutations is exponential

We considered whether the distribution of beneficial mutations fit a single distribution more than others tested. The distribution of beneficial mutations is better fit by an exponential curve than by a linear fit for both mutation collections of *B. cenocepacia* and *E. coli* (Fig. 2A-D). A higher fraction of the variance in three of the four collections, save the mutants of *B. cenocepacia* evolved in galactose, was explained by an exponential model than by the linear model. However, the standard error of the exponential fit was less than the standard error of the linear fit in all four collections, which supports the exponential model. It is interesting to note that both collections of *B. cenocepacia* mutants (Fig. 1A and B) achieved a higher relative fitness value than both collections of *E. coli* mutants (Fig. 2C and D).


(A) Distribution of Direct Effects for Mutants of B. cenocepacia selected in Galactose
Number of mutants
Relative Fitness
Mutants
Fit y linear
Fit y exponential
Exponential: S(y)= 0.87
r-squared= 0.22
Linear: S(y)= 0.94
r-squared= 0.32


(B) Distribution of Direct Effects for Random Tn5 Mutants of B. cenocepacia
Number of Mutants
Relative Fitness
Mutants
Fit y linear
Fit y exponential
Exponential: S(y)= 0.76
r-squared= 0.48
Linear: S(y)= 3.3
r-squared= 0.25



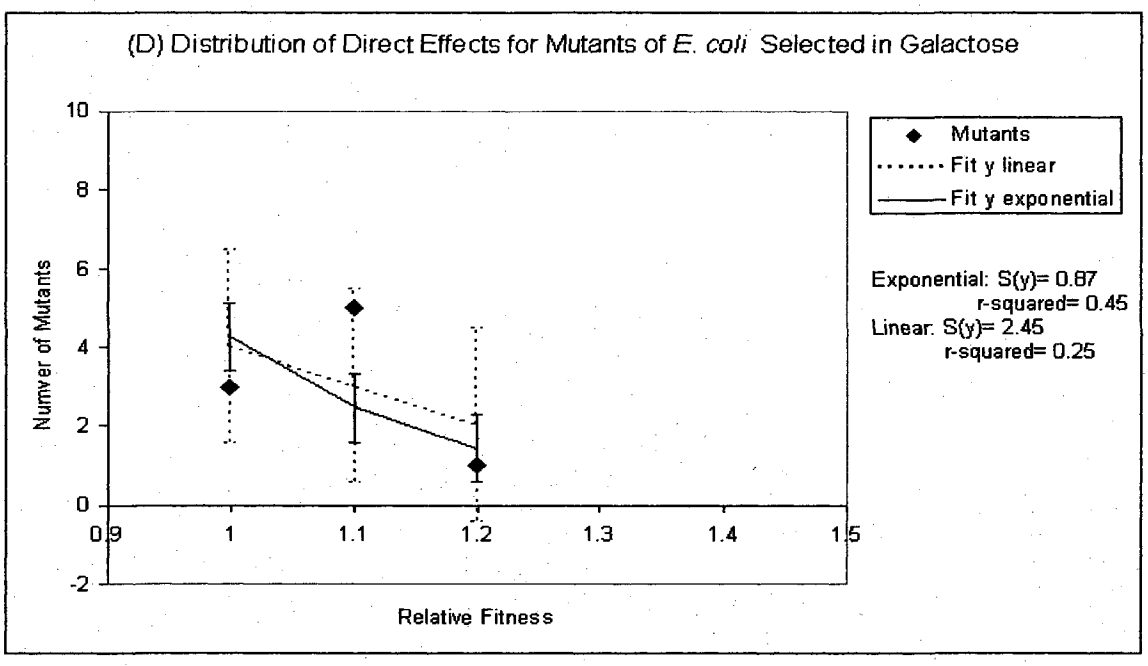
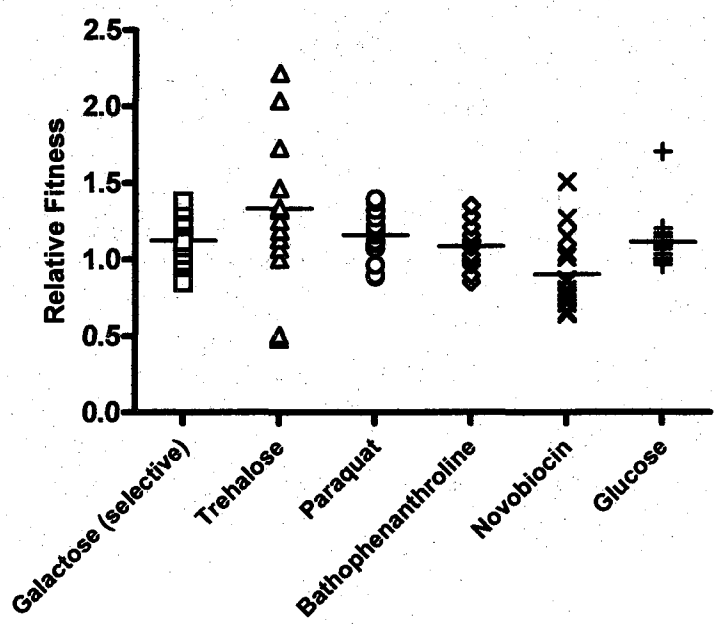


Figure 2. Histograms of beneficial mutations in their selective environment. All relative fitness estimates were binned into increments of 0.10. Relative fitness values less than 1.00 were excluded from the graphs and analyses. The dotted lines represent the linear fit using the least sum of squares method and the dotted error bars representing the standard error for the linear fit. The solid line represents the exponential fit using the least sum of squares and the solid error bars representing the standard error for the exponential fit. A) *B. cenocepacia* evolved in galactose, n=13 B) random Tn5 mutants of *B. cenocepacia*, n=14 C) *E. coli* mutants evolved in glucose, n=14 D) *E. coli* mutants evolved in galactose, n=9

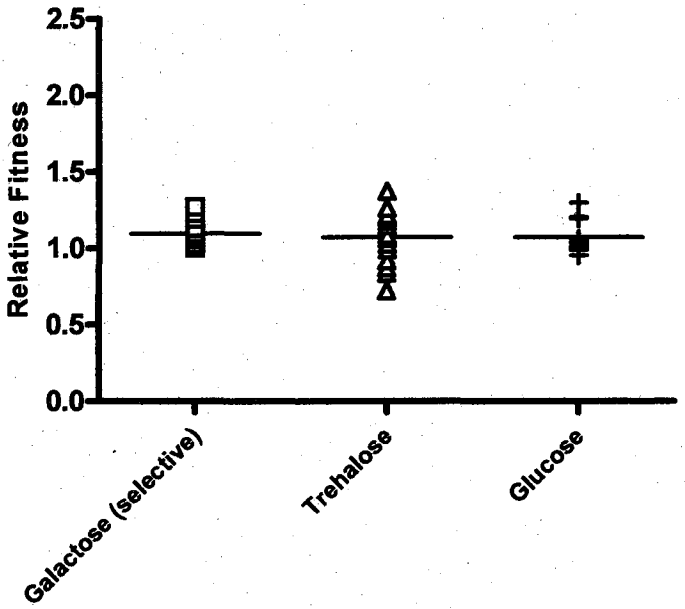
Pleiotropic effects are dependant on genotype and selective environment

Next, we considered if the distribution of pleiotropic effects varies between environments, genotypes, or both. We found that the distribution of pleiotropic effects varies greatly not only between selective environments, but also the different ancestral genotypes. The mutants of *B. cenocepacia* and *E. coli* evolved in galactose, while evolved in the same environment, exhibit different pleiotropic patterns in the same novel environments (Fig. 3A and C). The extent and pattern of pleiotropic effects also differed between mutants of the same ancestral genotype evolved in different environments (Fig. 3A and B and Fig. 3C and D).

(A) Mutants of *B. cenocepacia* Selected in Galactose



(B) Random Tn5 *B. cenocepacia* Mutants



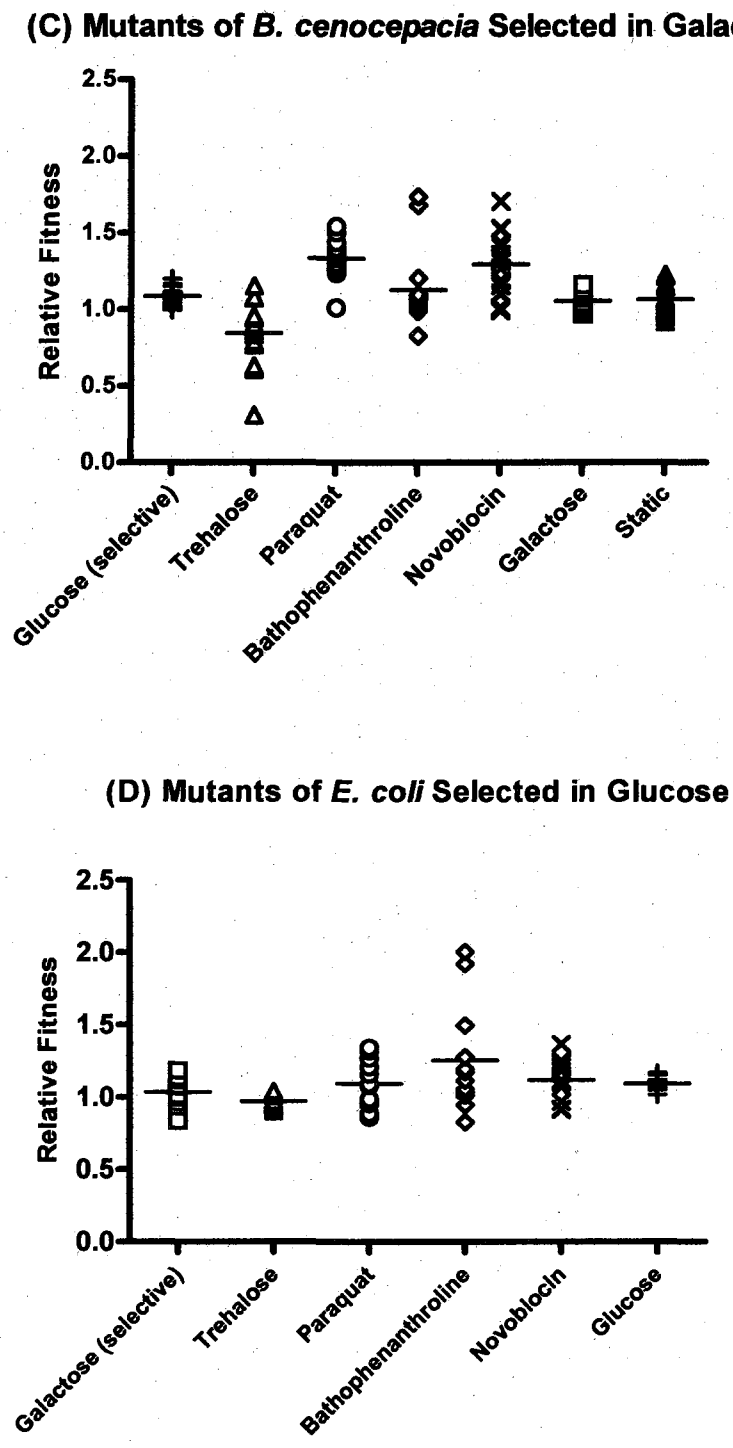


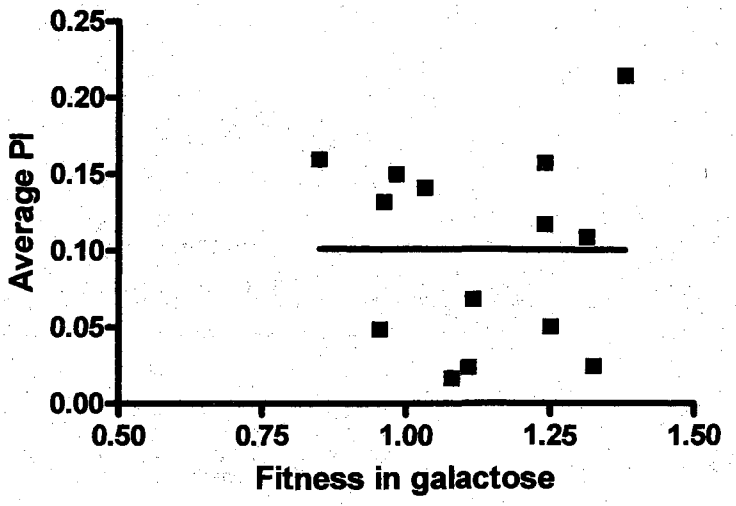
Figure 3. Relative fitness of independently derived mutants in selective and novel resources. Each shape represents the mean relative fitness of a mutant based on three independent measurements. The lines represent the mean fitness in each resource based on as n= A)13 mutants of *B. cenocepacia* selected for increased growth in galactose B) 14 random Tn5 mutants of *B. cenocepacia* with increased growth in galactose C) 13 mutants of *E. coli* selected for increased growth in glucose D) 10 mutants of *E. coli* selected for increased growth in galactose.

Different genotypes adapted in the same environment differ in their extent of pleiotropy

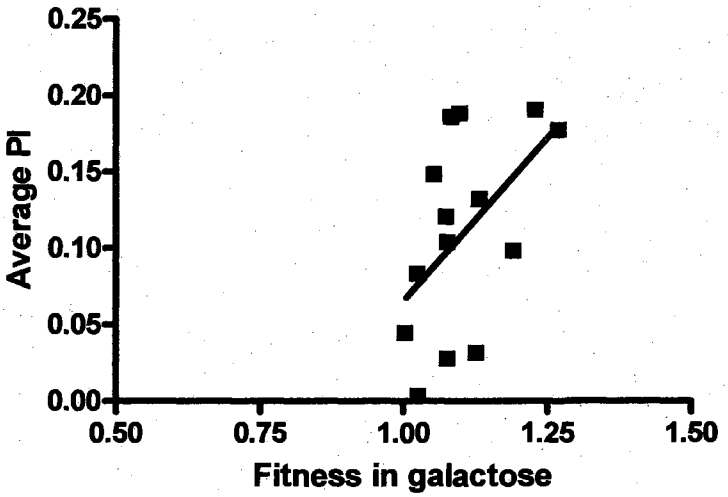
We then considered if different genotypes adapted to the same environment differed in the extent of pleiotropy, or if the genotypes all displayed the same pleiotropic effects. Fitness of each mutant collection in each foreign environment is summarized in the Appendix. These values are summarized as an overall pleiotropic index (PI) for each mutant and plotted as a function of the selective benefit of each mutant (Fig. 4D). By plotting the graphs this way, we have the ability to determine the overall form of pleiotropy, be it positive, negative or neutral. Among mutants of *E. coli* selected in galactose, the extent of pleiotropy is negative related to the magnitude of advantage in the selective environment (slope of the best-fit line is statistically different from zero, $p<0.0052$). Among the three other mutant collections, no such relationship between pleiotropy and selective benefit was found (slope of the best-fit line did not significantly deviate from zero) (Fig. 4A, B, and C). However, the overall extent of pleiotropy among mutant collections (the elevation of the slopes of direct versus pleiotropic effects) varied significantly ($F = 3.14545$, $df = 3$, $p<0.03418$).

When comparing the mutants of *B. cenocepacia* and *E. coli* evolved in galactose, the *B. cenocepacia* mutants were more pleiotropic than those of *E. coli*, or, in other words, mutants of *B. cenocepacia* tended to affect fitness in more environments than those of *E. coli*.

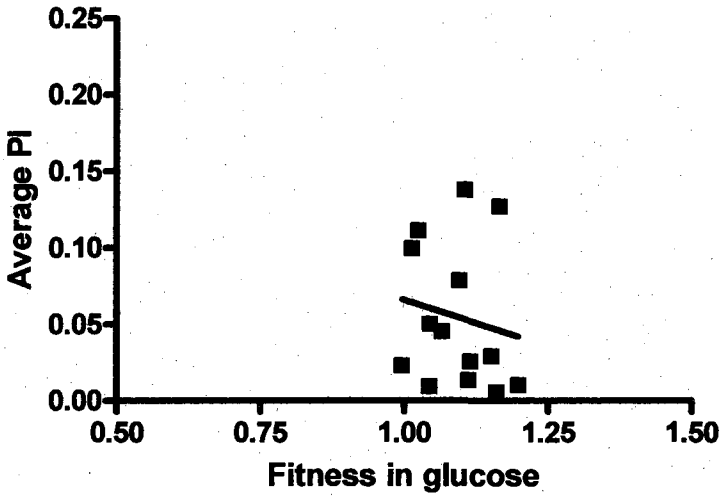
(A) Pleiotropic effects of *B. cenocepacia* mutants evolved in galactose



(B) Pleiotropic Effects of Tn5 Mutants of *B. cenocepacia*



(C) Pleiotropic Effects of *E. coli* Mutants Evolved in Glucose



(D) Pleiotropic Effects of *E. coli* Mutants Evolved in Galactose

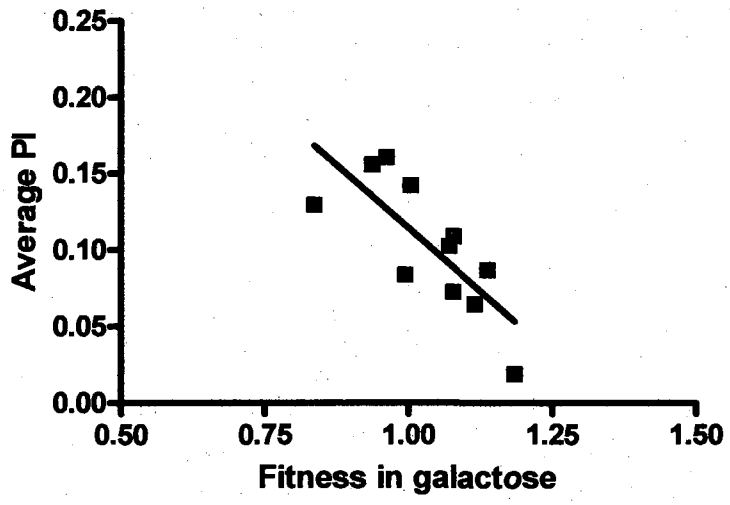


Figure 4. The relationship between fitness in the selective environment and the average pleiotropic index (PI) from multiple novel environments. The average PI for each mutant was then plotted against the relative fitness of the selective environment. A) mutants of *B. cenocepacia* selected for increased growth in galactose B) random Tn5 mutants of *E. cenocepacia* with increased growth in galactose C) mutants of *E. coli* selected for increased growth in glucose D) mutants of *E. coli* selected for increased growth in galactose.

We next calculated the grand mean PI for all mutants in each collection; this reflects the overall extent of pleiotropy among mutants originating under identical conditions (Fig. 5). Effects in galactose were relatively small and similar

to those observed in glucose, whereas the PI of other environments was greater and more variable (Fig. 5).

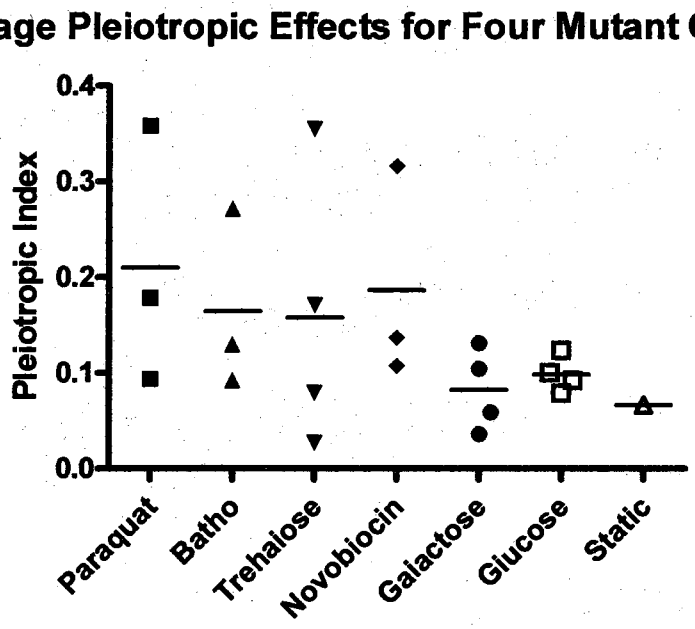


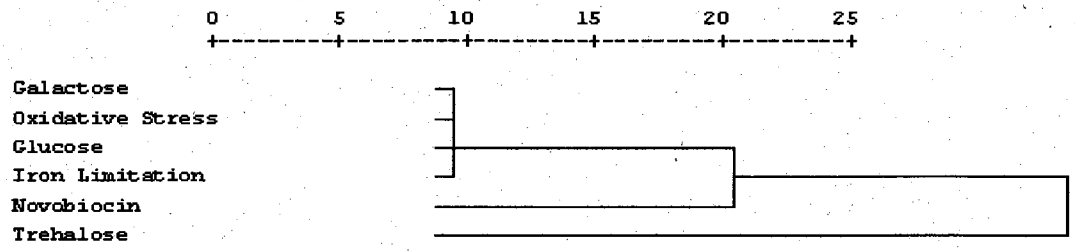
Figure 5. The average PI for each mutant collection in several novel environments. The average PI was found for each individual mutant in a mutant collection and then averaged together to get the average PI for the entire mutant collection. The average PI was then plotted with respect to the environments in which these data were collected.

Each collection of mutants adapted to the selective environment differently, resulting in diverse pleiotropic effects

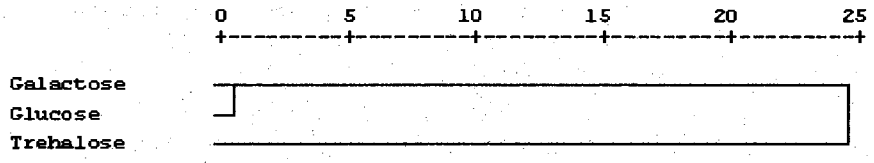
A hierarchical cluster analysis was used to group mutant collections by their fitnesses in multiple environments. The responses of each mutant collection differed in foreign environments (Fig. 6 A-D). All four sets of mutants produced similar responses in glucose and galactose whereas fitness in

trehalose and bathophenanthroline (iron limitation) were highly variable.

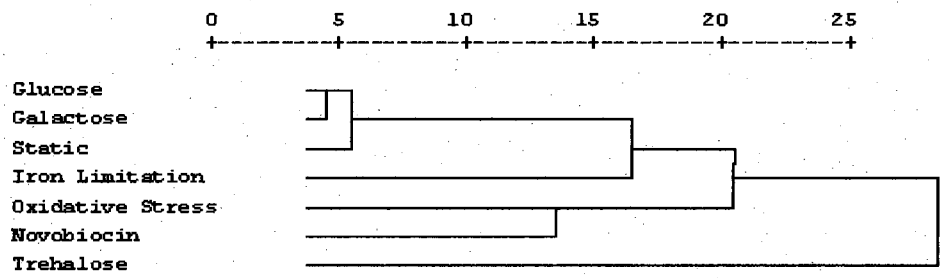
(A) Hierarchical Cluster Analysis of Mutants of *B. cenocepacia* Evolved in Galactose



(B) Hierarchical Cluster Analysis of Random Tn5 Mutants of *B. cenocepacia*



(C) Hierarchical Cluster Analysis of Mutants of *E. coli* Evolved in Glucose



(D) Hierarchical Cluster Analysis of Mutants of *E. coli* Evolved in Galactose

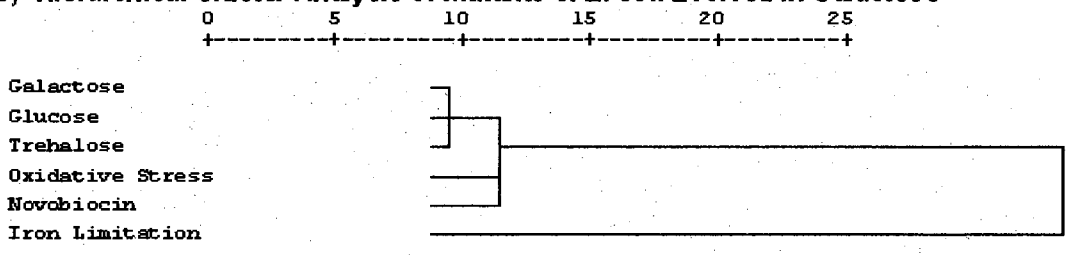


Figure 6. Hierarchical cluster analysis based on the relative fitness of independently derived mutants in novel environments. The distances between environments are a normalized Euclidean distance. A) 14 mutants of *B. cenocepacia* evolved in galactose in the selective environment and five novel environments B) 14 random Tn5 mutants of *B. cenocepacia* in two novel environments C) 13 mutants *E. coli* evolved in glucose in the selective environment and six novel environments D) 10 mutants of *E. coli* evolved in galactose in the selective environment plus five novel environments.

The only mutant collection that did not vary significantly among environments was not filtered by selection

All four collections of mutants produced significant genotype by environment interactions ($p<0.0001$). More specifically, the fitness of each mutant is dependent on the environment and the rank order of the fitness of each mutant is affected by the environment. In three of four mutant collections, mutant fitness on average varied with environment ($P<0.001$) (Table 1A, C, and D); however, random Tn5 mutants of *B. cenocepacia*, which were not filtered by selective pressure in the selective environment, performed similarly in each of the three environments tested ($P=0.788$) (Table 1B).

(A) Mutants of *B. cenocepacia* Evolved in Galactose

Source		d.f.	MS	F	P
Genotype (G)		13	0.335	1.742	0.073
Environment (E)		5	0.928	4.822	0.001
G x E		65	0.192	193.955	0.000
Error		168			

(B) Random Tn5 Mutant of *B. cenocepacia*

Source		d.f.	MS	F	P
Genotype (G)		13	0.080	2.232	0.039
Environment (E)		2	0.009	0.241	0.788
G x E		26	0.036	86.108	0.000
Error		84			

(C) Mutants of *E. coli* Evolved in Glucose

Source		d.f.	MS	F	P
Genotype (G)		12	0.123	0.694	0.751
Environment (E)		5	1.143	6.639	0.000
G x E		60	0.177	51.737	0.000
Error		174			

(D) Mutants of *E. coli* Evolved in Galactose

Source	d.f.	MS	F	P
Genotype (G)	9	.707	146.484	.000
Environment (E)	5	.581	120.371	.000
G x E	45	.298	61.688	.000
Error	138			

Table 1. ANOVA performed on the fitness effects of independently derived mutations of *B. cenocepacia* and *E. coli*. A) 14 mutants of *B. cenocepacia* evolved in galactose in five novel environments B) 14 random mutants of *B. cenocepacia* in two novel environments C) 13 mutants of *E. coli* evolved in glucose in five novel environments D) 10 mutants of *E. coli* evolved in galactose in five novel environments.

CHAPTER IV

DISCUSSION

The distribution of beneficial mutations as well as the distribution of pleiotropy affects not only how mutations shape a population, but how a population responds to changes in the surrounding environment (Sanjuan et al., 2004a, Wahl et al., 2002, Colosimo et al., 2005, Cooper, 2002, Cooper et al., 2001, Williams, 1957, Lenski, 1998, Otto, 2004). The work presented here pursued three distinct objectives. The first was to define the distribution of beneficial mutations for four collections of mutants. Whereas the distribution of beneficial mutations has been previously characterized by a few methods and generally found to be exponential (Rozen et al., 2002, Kassen & Bataillon, 2006, Imhof & Schlotterer, 2001) except for a recent study by Rokyta et al. (Rokyta et al., 2008) these studies only examined beneficial mutations arising in a single genetic background in a single selective environment. By examining mutants in different environments and in different genetic backgrounds, we can address how environment and genotype affect the distribution of beneficial alleles. Our results support the theoretical studies that state the higher the fitness of a mutation, the rarer it will be in the population, and also seem to suggest an upper limit to adaptive steps (a right-truncated distribution), as described by Roykta et al. 2008 (Rokyta et al., 2008) (Fig. 1 A-D). We also find that mutations of less well

adapted organisms (the *B. cenocepacia* ancestor is naïve to the laboratory, and our *E. coli* ancestor is not) tend to have a greater selective benefit, as predicted by Fisher's fundamental theorem (Fisher 1930) (Fig. 1A and B). The fact that the isolate of *E. coli* used in this study was unable to achieve the same fitness levels as the *B. cenocepacia* is evidence that the *E. coli* isolate has undergone previous adaptation and had now a smaller pool of beneficial mutations to choose from. Adaptation to an environment is the movement of a population towards a phenotype that best fits the present environment, and because *B. cenocepacia* is further away from its optimum, it had greater room for improvement (Fisher, 1930).

The second objective was to describe the overall form of pleiotropic effects for *E. coli* and *B. cenocepacia*. In theory, pleiotropy may be positive or antagonistic and may associate with the magnitude of direct mutational effects in either direction. The commonly held 'tradeoff model' suggests that as the benefit of favored mutations increases, so too does the antagonistic effects of these mutations in different environments. We quantified the form of pleiotropy by measuring fitness of each mutant in a range of foreign environments differing in carbon source or the presence of stressors (Fig. 2A-D). Somewhat surprisingly, we found that in novel stressful environments (environments the organism has not encountered while undergoing selection), the predominant form of pleiotropy is positive, though some individual mutants exhibit strong antagonism. Despite assays of fitness in several stressful environments, antagonistic pleiotropy was only consistently detected for a single genotype-by-environment combination (*E.*

coli in trehalose) (Fig. 2C and D). These results indicate that the first steps of adaptation tend to improve general vigor and increase fitness in many environments, and suggest that the ecological specialization observed during longer-term adaptation (Futuyma & Moreno, 1988, Cooper & Lenski, 2000, Cooper et al., 2001, Cooper, 2002) may be caused instead by pleiotropic effects of secondary mutations or epistasis among beneficial alleles.

Our results also address whether adaptive mechanisms vary among mutants within a genotype/environment combination and between distinct genotypes or environments. Returning to the environment with trehalose as sole carbon source, both collections of *B. cenocepacia* mutants were on average better than the ancestor and the same or better than they were in the selective environment (galactose) (Fig. 2A and B). This implies that most mutants of *B. cenocepacia* that are adaptive in galactose also enhance metabolism of other resources. Yet this was not always the case: certain mutants of *B. cenocepacia* grown in trehalose were the least fit of all mutant-by-environment combinations measured. How can we explain this wide variability in pleiotropic effects? Trehalose and glucose are both transported across the inner membrane by the phosphotransferase system (PTS), but they traverse the outer membrane by different proteins, LamB and OmpF, respectively. Galactose, on the other hand, is not a PTS sugar, but it uses the same OmpF protein as glucose to cross the membrane (Travisano & Lenski, 1996). Because some mutants of both *B. cenocepacia* and *E. coli* selected in galactose had decreased fitness in trehalose, they may have acquired mutations that affected LamB-mediated transport in

favor of OmpF-mediated transport. Alternatively, trehalose metabolism may have been compromised because of its influence on membrane fluidity and its role in resistance to stress induced by low temperature (Horlacher & Boos, 1997, Liu et al., 2000, Kandror et al., 2002), traits presumably irrelevant when selection favors efficient transport of different sugars at moderate temperature. One of the mutant collections used for this thesis, mutations of *E. coli* evolved in glucose, was sequenced using a candidate gene approach with the selection of five genes based previous research. The authors found that out of the 27 mutants sequenced, 13 of the mutants had mutations in spoT, 5 had mutations in the nadR, one of each hokB/sokB, pbpA-rodA, pykF, and 7 mutants had unknown mutations, due to the fact that a mutation was not found in any of the candidate genes (Ostrowski et al., 2007). These results suggest that mutations in global regulators rather than cell wall mutations, are under the greatest amount of selective pressure in a carbon source limiting environment. Furthermore, the wide variation among *B. cenocepacia* mutants and low variation among *E. coli* mutants favored in galactose medium, but evaluated in trehalose medium, suggests that genetic background alone can profoundly influence the extent of pleiotropy among favored mutations (Fig. 2A and D).

The third objective for this thesis was to define the relationship between the direct benefits of selected mutations and their indirect effects in foreign environments. A significant relationship between these two measures was found in only one collection of mutants (*E. coli* selected in galactose), and the relationship was negative. *E. coli* selected in glucose also showed a negative

relationship, but the slope is not statistically different from zero. In the collection of *B. cenocepacia* mutants evolved in galactose, no relationship between direct and indirect effects was evident, but in the collection of random Tn5 mutants, the relationship between direct and indirect effects appears positive, but the slope is not statistically different from zero (Fig. 3A-D).

We also found that the mode of mutant collection may influence the extent of variation in fitness among environments (Fig. 4). Prior studies have employed two different approaches to characterize pleiotropic effects of mutations: i) random or directed mutagenesis and minimal selection, and ii) selection of naturally occurring mutations in a defined environment. The first approach potentially generates a large number of independent mutations but their limited exposure to selection and unnatural origin means that it is unrealistic to assume that these mutations would be detectable outside the laboratory. The second approach subjects an organism to a single environment and mutants are collected when they exhibit increased growth when compared to the ancestor. This type of study allows mutations to be filtered by selection, but the effects of only a subset of possible mutations can be studied. This is the first study to employ both methods and compare their outcomes directly. Notably, we find that the sensitivity of mutations to different environments (the GxE of the mean of each collection) is much greater when mutants are collected by 'natural' selection (the second method) than when generated artificially (the first method). This implies that the types of mutations caused by transposons may inherently differ from those that occur during the adaptive process and generally enhance growth

irrespective of environment, whereas selection may favor more diverse mutations with greater environment specificity (Table 1).

Our results shed light not only on the distribution of beneficial mutations but also their effects in other environments. We find substantial effects of genotype, environment, and the mode of mutant selection. However, much work remains to be done before we gain a more complete picture of the pleiotropic effects of beneficial mutations and how genotype-by-environment interactions evolve. First, collecting more mutants in each of the four environments will expand our chances of collecting mutations with greater and lesser benefit. Second, to better understand how the selective environment influences the pleiotropy of favored mutants, the current collections of mutants should be compared with the following: mutants of *B. cenocepacia* evolved in glucose and in trehalose and mutants of *E. coli* evolved in trehalose and derived from transposon mutagenesis. These collections balance our current design in a statistical sense and address whether the antagonistic pleiotropy of glucose- or galactose-selected mutants grown in trehalose is reciprocal (i.e. are trehalose-selected mutants antagonistic for growth in galactose). Further, to study whether pleiotropic effects of selected mutations increase during adaptive walks towards the selective optimum (Fisher 1930), we must collect mutants of intermediately adapted genotypes. Thus, an isolate of *B. cenocepacia*, having been evolved for 1000 generations in galactose, and an isolate of *E. coli*, having been evolved for 2000 generations in glucose, are suitable ancestors for such an approach. These experiments will enable us to observe how the selective history of an

organism influences the distribution of beneficial and pleiotropic effects. We may also wish to measure indirect effects in additional stressful environments to determine whether positive pleiotropy is ubiquitous. Such environments might include antibiotics affecting different targets, growth in low and high temperatures, those affecting both outer and inner membrane function, and other general stressors to the cell. We anticipate that studies in additional foreign environments may target different sets of functions than those favored by the selective environment and yield different forms of pleiotropy. Finally, future work should be focused on identifying the genetic mechanisms of each of the mutant collections, which is now quite feasible by complete genome resequencing. The underlying adaptive mutations of one of these collections, *E. coli* evolved in glucose, have already been mostly had identified (Ostrowski et al., 2007). These results provide several candidate genes in which we may begin our search for mutations. In the future, the large amount of genome sequence data may be integrated with a range of physiological information, which will identify the precise targets of selection and their impacts on the global physiological phenotype and ecological limits of evolving populations.

LIST OF REFERENCES

Barton, N. 1990. Pleiotropic models of quantitative variation. *Genetics* **124**: 773-782.

Bertani, G. 1951. The mode of phage liberation by lysogenic Escherichia coli. *Journal of Bacteriology* **62**: 293-300.

Braun, V. 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. *Biol. Chem.* **378**: 779-786.

Bulmer, M. G. 1973. The maintenance of the genetic variability of polygenic characters by heterozygous advantage. *Genet. Res., Camb.* **22**: 9-12.

Burkholder, W. 1950. Sour skin, a bacterial rot of onion bulbs. *Phytopathology*: 468-475.

Colosimo, P. F., Hosemann, K. E., Balabhadra, S., Villarreal, G. J., Dickson, M., Grimwood, J., Schmutz, J., Myers, R. M., Schluter, D. & Kingsley, D. 2005. Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin allels. *Science* **307**: 1928-1933.

Cooper, T. F., Rozen, D. E. & Lenski, R. E. 2002. Parallel changes in gene expression after 20,000 generations of evolution in *Escherichia coli. PNAS* **100**: 1072-1077.

Cooper, V. S. 2002. Long-term experimental evolution in *Escherichia coli.* X. Quantifying the fundamental and realized niche. *BMC Evolutionary Biology* **2**.

Cooper, V. S. & Lenski, R. E. 2000. The population genetics of ecological specialization in evolving *Escherichia coli. Nature* **407**.

Cooper, V. S., Schneider, D., Blot, M. & Lenski, R. E. 2001. Mechanisms causing rapid and parallel losses of ribose catabolism in evolving populations of *Escherichia coli* B. *Journal of Bacteriology* **183**: 2834-2841.

Cowart, R., Singleton, F. & Hind, J. 1993. A comparison of bathophenanthrolinedisulfonic acid and ferrozine as chelators of iron (II) in reduction reactions. *Anal Biochem* **211**: 151-155.

Cunningham, C. W., Jeng, K., Husti, J., Badgett, M., Molineaux, I. J., Hillis, D. M. & Bull, J. J. 1997. Parallel molecular evolution of deletions and nonsense mutations in bacteriophage T7. *Mol. Biol. Evol.* **14**: 113-116.

Donnelly, A. & Blagg, B. 2008. Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket. *Curr Med Chem* **15**: 2702-2717.

Dudley, A. M., Janse, D. M., Tanay, A., Shamir, R. & Church, G. M. 2005. A global view of pleiotropy and phenotypically derived gene function in yeast. *Molecular Systems Biology*.

Ferris, M. T., Joyce, P. & Burch, C. L. 2007. High frequency of mutations that expand the host range of an RNA virus. *Genetics* **176**: 1013-1022.

Fisher, R. 1930. *The genetical theory of natural selection*. Oxford University Press, Oxford, UK.

Futuyma, D. J. & Moreno, G. 1988. The evolution of specialization. *Annual Review of Ecology and Systematics* **19**: 207-233.

Gerrish, P. J. & Lenski, R. E. 1998. The fate of competing beneficial mutations in an asexsual population. *Genetica* **102/103**: 127-144.

Gil, F., Ipinza, F., Fuentes, J., Fumeron, R., Villarreal, J. M., Aspee, A., Mora, G. C., Vasquez, C. C. & Saavedra, C. 2007. The *ompW* (porin) gene mediates methyl viologen (paraquat) efflux in *Salmonella enterica* serovar Typhimurium. *Research in Microbiology* **158**: 529-536.

Gillespie, J. 1983. A simple stochastic gene substitution model. *Theor. Popul. Biol.* **23**: 202-215.

Gillespie, J. 1984. Molecular evolution over the mutational landscape. *Evolution* **38**: 1116-1129.

Griswold, C. K. & Whitlock, M. C. 2003. The genetics of adaptation: the roles of pleiotropy, stabilizing selection and drift in shaping the distribution of bidirectional fixed mutational effects. *genetics* **165**: 2181-2192.

Gumbel, E. J. 1958. *Statistics of extremes*. Univ. Press, Oxford.

Hegreness, M., Shoresh, N., Hartl, D. & Kishony, R. 2006. An equivalence principle for the incorporation of favorable mutations in asexual populations. *Science* **311**: 1615-1617.

Heineman, R. H. & Bull, J. J. 2007. Testing optimality with experimental evolution: Lysis time in a bacteriophage. *Evolution* **61**: 1695-1709.

Horlacher, R. & Boos, W. 1997. Characterization of TreR, the major regulator of the *Escherichia coli* trehalose system. *J Biol Chem* **20**: 13026-32.

Imhof, M. & Schlotterer, C. 2001. Fitness effects of advantageous mutations in evolving *Escherichia coli* populations. *Proceedings of the National Academy of Science* **98**: 1113-1117.

Isles, A., Macklusky, I., Corey, M., Gold, R., Prober, C., Fleming, P. & Levison, H. 1984. Pseudomonas cepacia infection in cystic fibrosis: An emerging problem. *The Journal of Pediatrics* **104**: 206-210.

Kandror, O., DeLeon, A. & Goldberg, A. L. 2002. Trehalose synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. *Proceedings of the National Academy of Science* **99**: 9727-9732.

Kassen, R. & Bataillon, T. 2006. Distribution of fitness effects among beneficial mutations before selection in experimental populations of bacteria. *Nature Genetics* **38**.

Klein, W. & Boos, W. 1993. Induction of the lambda receptor is essential for effective uptake of trehalose in *Echerichia coli*. *Journal of Bacteriology* **175**: 1682-1686.

Lamont, I. L., Beare, P. A., Ochsner, U., Vasil, A. I. & Vasil, M. L. 2002. Siderophore-mediated signaling regulates virulence factor production in Pseudomonas aeruginosa. *Proceedings of the National Academy of Science* **99**: 7072-7077.

Lande, R. 1980. The genetic covariance between characters maintained by pleiotropic mutations. *Genetics* **94**: 203-215.

Leadbetter, M. R., Lindgren, G. & Rootzen, H. 1983. *Extremes and related properties of random sequences and processes*. Springer, New York.

Lenski, R. E. 1998. Bacterial evolution and the cost of antibiotic resistance. *International Microbiology* **1**: 265-270.

Lenski, R. E. 2004. Phenotypic and genomic evolution during a 20,000 generation experiment with the bacterium *Escherichia coli*. *Plant Breeding Reviews* **24**: 225-265.

Lenski, R. E. (2009) The *E. coli* long-term experimental evolution project site. pp.

Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. 1991. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *American Naturalist* **138**: 1315-1341.

LiPuma, J. J., Spilker, T., Coenye, T. & Gonzalez, C. F. 2002. An epidemic *Burkholderia cepacia* complex strain identified in soil. *The Lancet* **359**: 2002-2003.

Liu, X., Ng, C. & Ferenci, T. 2000. Global adaptations resulting from high population densities in *Escherichia coli* cultures. *J Bacteriol.* **183**: 4158-4164.

Mdluli, K. & Zhenkun, M. 2007. Mycobacterium tuberculosis DNA gyrase as a target for drug discovery. *Infect Disord Drug Targets* **7**: 159-168.

Miralles, R., Gerrish, P. J., Moya, A. & Elena, S. F. 1999. Clonal interference and the evolution of RNA viruses. *Science* **285**: 1745-1747.

O'Sullivan, L. A. & Mahenthiralingam, E. 2005. Biotechnological potential within the genus *Burkholderia*. *Lett Appl Microbiol*: 8-11.

Orr, H. A. 2003. The distribution of fitness effects among beneficial mutations. *Genetics* **163**: 1519-1526.

Ostrowski, E. A., Rozen, D. E. & Lenski, R. E. 2005. Pleiotropic effects of beneficial mutations in *Escherichia coli*. *Evolution* **59**: 2343-2352.

Ostrowski, E. A., Woods, R. J. & Lenski, R. E. 2007. The genetic basis of parallel and divergent phenotypic responses in evolving populations of *Escherichia coli*. *Proceedings of the Royal Society of Biology*.

Otto, S. P. 2004. Two steps forward, one step back: The pleiotropic effects of favoured alleles. *Proceedings of the Royal Society of Biology* **271**: 705-714.

Peichel, C. 2005. Fishing for the secrets of vertebrate evolution in threespine sticklebacks. *Dev. Dyn.* **234**: 815-823.

Riley, M. S., Cooper, V. S., Lenski, R. E., Forney, L. J. & Marsh, T. L. 2001. Rapid phenotypic change and diversification of a soil bacterium during 1000 generations of experimental evolution. *Microbiology* **147**: 995-1006.

Rokyta, D. R., Beisel, C. J., Joyce, P., Ferris, M. T., Burch, C. L. & Wichman, H. A. 2008. Beneficial fitness effects are not exponential for two viruses. *J Mol Evol* **67**: 368-376.

Rozen, D. E., deVisser, J. A. G. M. & Gerrish, P. J. 2002. Fitness effects of fixed beneficial mutations in microbial populations. *Current Biology* **12**: 1040-1045.

Sanjuan, R., Moya, A. & Elena, S. F. 2004a. The contribution of epistasis to the architecture of fitness in an RNA virus. *Proceedings of the National Academy of Science* **101**: 15376-15379.

Sanjuan, R., Moya, A. & Elena, S. F. 2004b. The distribution of fitness effects caused by single-nucleotide substitutions in an RNA virus. *Proceedings of the National Academy of Science* **101**: 8396-8401.

Segre, A. V., Murry, A. W. & Leu, J.-Y. 2006. High-resolution mutation mapping reveals parallel experimental evolution in yeast. *PLoS Biology* **4**: 1372-1385.

Travisano, M. & Lenski, R. E. 1996. Long-term experimental evolution in *Escherichia coli*. IV. Targets of selection and the specificity of adaptation. *Genetics* **143**: 15-26.

Turner, P. E. & Chao, L. 2003. Escape from prisoner's dilemma in RNA phage phi 6. *American Naturalist* **161**: 497-505.

Vanlaere, E., Baldwin, A., Gevers, D., Henry, D., De Brandt, E., LiPuma, J. J., Mahenthiralingam, E., Speert, D. P., Dowson, C. G. & Vandamme, P. 2009. Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **59**: 102-111.

Velicer, G. J., Raddatz, G., Keller, H., Deiss, S., Lanz, C., Dinkelacker, I. & Schuster, S. 2006. Comprehensive mutation identification in an evolved bacterial cooperator and its cheating ancestor. *Proceedings of the National Academy of Science* **103**: 8107-8112.

Wahl, L. M., Gerrish, P. J. & Saika-Voivod, I. 2002. Evaluating the impact of population bottlenecks in experimental evolution. *Genetics* **162**: 961-971.

Wichman, H. A., Badgett, M. R., Scott, L. A., Boulianne, C. M. & Bull, J. J. 1999. Different trajectories of parallel evolution during viral adaptation. *Science* **285**: 422-424.

Wilke, C. 2004. The speed of adaptation in large asexual populations. *Genetics* **167**: 2045-2053.

Williams, G. C. 1957. Pleiotropy, natural selection and the evolution of senescence. *Evolution* **11**: 398-411.

APPENDIX

Table 2. Average fitness effects of each mutant collection in all environments tested. Each number is the mean of three replicates. The bold numbers represent the fitness of each mutant in the selective environment.

	Glucose	Trehalose	Paraquat	Batho	Novobiocin	Galactose	Static
E. coli Evolved in Glucose							
REL 10016	**0.996074**	0.607156	1.5419702	1.205399	1.5245013	0.970375	0.96997
REL 10012	**1.01374**	0.886263	1.2827631	0.989373	1.1079491	1.004944	0.918194
REL 9982	**1.023897**	0.957886	1.5025095	1.736854	1.3585084	1.043738	1.215601
REL 9994	**1.043885**	0.834854	1.2355111	1.072343	1.7053611	1.159593	1.024425
REL 10020	**1.04475**	0.630533	1.3506118	1.022682	1.3112366	1.073244	1.164767
REL 10004	**1.065262**	0.768602	1.3670878	1.048983	1.2697395	1.003967	1.112788
REL 9992	**1.095771**	0.881322	1.2694303	0.827269	1.1892614	1.096608	1.063517
REL 10018	**1.10545**	0.309814	1.3335678	1.048163	1.0110512	1.018961	1.06849
REL 9996	**1.111343**	1.154063	1.2592554	1.063766	1.319039	0.986337	0.996838
REL 9980	**1.114713**	1.079718	1.4002124	1.034914	1.1004035	1.115788	0.94749
REL 9974	**1.15136**	0.857886	1.3796818	0.997407	1.1549749	0.995018	1.22837
REL 10000	**1.160022**	0.90361	1.3487248	1.0107	1.4367671	1.113646	1.072802
REL 10006	**1.165746**	0.942609	1.4384326	1.6798	1.530841	1.111917	1.146506
REL 10014	**1.198595**	0.788117	1.3036459	1.081612	1.4084321	1.130876	1.002182
E. coli Evolved in Galactose							
E9 (-)	1.053983	1.028218	0.859993	1.032031	1.208619	**0.83664**	
G12 (+)	1.109974	0.922303	0.952706	1.11429	0.965323	**0.936866**	
H9 (+)	1.168371	1.027595	0.976742	0.944861	0.91762	**0.961698**	
B12 (-)	1.094663	1.03878	1.3428	0.825656	1.179723	**0.994875**	
E9 (+)	1.070781	0.919255	1.078446	1.010136	1.06738	**1.003469**	
C10 (-)	1.049153	0.926491	0.98562	1.192133	1.231492	**1.071012**	
A9 (-)	1.058572	0.9689	1.207876	1.171739	1.098582	**1.07828**	
F10	1.153019	0.9008	1.267375	2.003929	1.365731	**1.078646**	
D11 (-)	1.116775	1.002247	1.15653	1.920716	1.249578	**1.115331**	
H11 (-)	1.116865	0.938673	0.886978	1.495357	1.103229	**1.137124**	
A11 (-)	1.109997	1.024953	1.31805	1.275901	1.113224	**1.184981**	
B. cenocepacia Evolved in Galactose							
A5 -	1.032474	0.482321	0.8982243	1.112596	1.50655	**0.848429**	
A7R +	1.115362	1.323499	1.2214583	1.029072	1.0147199	**0.954564**	
F6 -	0.966917	1.465776	0.9656338	0.966646	0.749358	**0.961259**	
A5 R +	1.002195	1.132828	1.0759705	0.897141	0.8577171	**0.983173**	
A7 -	1.083425	1.070035	1.1059364	0.853277	0.8668186	**1.032525**	
D7 +	1.169742	1.188329	1.3752007	1.351337	0.7188693	**1.079637**	
F6 +	1.087096	1.727334	1.1223348	1.124279	0.6624704	**1.108796**	
C6 +	1.067795	1.187107	1.2737792	1.122207	0.7546988	**1.116404**	
H7 +	1.147418	2.03613	1.3243397	1.082404	1.2695412	**1.240205**	
E5 R +	1.095274	0.505802	1.1930501	0.993772	1.1512478	**1.240226**	
H5 +	0.965733	1.254128	1.1625566	1.211671	0.8032848	**1.244069**	
E5 S +	1.201024	2.036202	1.2133439	1.281326	0.7152884	**1.313998**	

A5 S+	1.090483	1.338913	1.1716043	1.116278	0.7861688	**1.325045**
A7 S +	1.702039	2.21728	1.3975076	1.15716	0.6443491	**1.380238**
Tn5 Mutants of *B. cenocepacia*						
D9 (-)	0.991364	0.872138				**1.002667**
G12 (-)	1.003719	0.721891				**1.024056**
E2 (-)	0.955802	1.028243				**1.025977**
F3 (-)	1.017944	1.374439				**1.052856**
F1 (-)	1.201999	1.086818				**1.0736**
A3 (-)	1.033626	1.202421				**1.07586**
F5 (-)	1.004742	0.835582				**1.076371**
G5 (-)	1.299621	1.176082				**1.082188**
D4 (-)	1.204382	1.262865				**1.097506**
A12 (-)	1.048978	0.91961				**1.126624**
B11 (-)	1.063059	1.201928				**1.131794**
G2 (-)	1.039389	1.065272				**1.190389**
H1 (-)	1.195046	1.148724				**1.228585**
H11 (-)	1.044555	1.218388				**1.268765**